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# Annual Viral Expression in a Sea Slug Population: Life Cycle Control and Symbiotic Chloroplast Maintenance

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*In a few well-known cases, animal population dynamics are regulated by cyclical infections of protists, bacteria, or viruses. In most of these cases, the pathogen persists in the environment, where it continues to infect some percentage of successive generations of the host organism. This persistent re-infection causes a long-lived decline, in either population size or cycle, to a level that depends upon pathogen density and infection level (1–4). We have discovered, on the basis of 9 years of observation, an annual viral expression in* Elysia chlorotica, *an ascoglossan sea slug, that coincides with the yearly, synchronized death of all the adults in the population. This coincidence of viral expression and mass death is ubiquitous, and it occurs in the laboratory as well as in the field. Our evidence also suggests that the viruses do not re-infect subsequent generations from an external pathogen pool, but are endogenous to the slug. We are led, finally, to the hypothesis that the viruses may be involved in the maintenance of symbiotic chloroplasts within the molluscan cells.*

Populations of the ascoglossan sea slug *Elysia chlorotica* occur in salt marshes from the Chesapeake Bay to Nova Scotia. The life cycle of the slug lasts about 10 months. The hermaphroditic adults lay egg masses in the spring of each year, and all of the adults die shortly afterward (5, 6). A week or so after the egg deposition, veliger larvae hatch. These larvae spend a few weeks in the plankton and, if filaments of the chromophytic alga *Vaucheria litorea* are present, each veliger homes in on one of them and attaches to it. During the next 24 h, the larva metamorphoses into a juvenile slug while still attached to the algal filament. If the algal filaments are not present, metamorphosis rarely occurs, at least not in laboratory cultures (5, 6). *Vaucheria* is the only alga that *E. chlorotica* eats, and it is the source of the symbiotic chloroplasts that are acquired during feeding. The juvenile slug immediately begins eating the algal filaments and taking on its first load of chloroplasts, which are sequestered by specialized cells in the epithelium lining the digestive diverticula (5, 6). During the next several months, the slugs continue to eat *Vaucheria* and grow, until winter temperatures cause them to become inactive. As the salt marshes warm in the spring, the slugs become active again, begin laying egg masses, and then die. By the time the egg masses have hatched in May, all the adults are gone. This mass mortality occurs synchronously in the laboratory as well as in the field and regardless of the time of year that the slugs were collected.

Symbioses in which chloroplasts—usually from a particular species of alga—are taken up and retained within the cytoplasm of an animal cell occur in several phyla, but they are most commonly encountered in molluscan sea slugs, particularly in the order Ascoglossa (=Sacoglossa) (Opisthobranchia). Certain of the molluscan cells can capture chloroplasts from algal food (usually from a specific species of either Rhodophyceae or Chlorophyceae), and these organelles retain some degree of photosynthetic function for a time (*e.g.*, 7, 8, 9). Whether this intracellular association is a symbiosis in the strict sense is debatable; some authors prefer terms like chloroplast symbiosis, chloroplast retention, or kleptoplasty (7, 9, 11, 12, 13), which indicate that the benefit of the association is entirely to the animal.

The duration of the association between the molluscan cell and the algal plastid varies from species to species.

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Some associations last less than a week [*e.g., Hermaea bifida* and *Elysia hedgpethi* (see 14)]; and although the plastids are initially functional, photosynthesis stops or is greatly reduced after a week of starvation. In contrast, plastid function continues for more than a week in starved specimens of several species, most belonging to the ascoglossan family Elysiidae (*e.g.*, 7, 10, 15, 16). In the species with the longer duration symbioses, the algal chloroplasts within the molluscan cells fix $^{14}CO_2$, and the $^{14}C$ appears in a variety of compounds in the animal tissues (15, 17, 18, 19, 20, 21). Thus, there is no doubt that the captured chloroplasts are photosynthetically active within the molluscan cell cytoplasm, and that the products of the synthesis are utilized by the host animal. Indeed, once the symbiosis is established, the species with the longer-lived chloroplast associations can be starved and, as long as light is provided, will maintain or actually gain body weight until the chloroplast function finally fails (7).

In plant cells, continued photosynthetic activity requires continuous synthesis of chloroplast proteins because several proteins, including those used in light harvesting, are rapidly turned-over during the process and must be replaced (22, 23, 24). But, several of these photosynthetic proteins (or their subunits) are encoded in the nucleus, so plastid protein synthesis requires the integration of two distinct genomes, that of the chloroplast and that of the plant cell nucleus (reviewed in 25). Because normal plastid photosynthetic function is dependent upon major nuclear and cytoplasmic support, the ultimate failure of photosynthesis by the symbiotic chloroplasts within the molluscan cells is not surprising. But the symbiotic plastids of *E. chlorotica* stay photosynthetically functional for 8–9 months (5, 26)—many months longer than those of any other species yet described. This remarkable persistence of chloroplast function during starvation indicates that replacement of at least the essential photosynthetic proteins must be occurring within the plastid while it is housed in the molluscan cytoplasm; and indeed, synthesis of plastid proteins, primarily those associated with photosynthesis, occurs in the *E. chlorotica* plastids (26, 27, 28).

The plastid proteins synthesized within the cells of *E. chlorotica* are of two pharmacologically distinguishable sorts: those whose synthesis is blocked by chloramphenicol, and those blocked by cycloheximide (26). Protein synthesis on *plastid* ribosomes is blocked by chloramphenicol, whereas synthesis on *cytoplasmic* ribosomes, usually directed by nuclear genes, is blocked by cycloheximide. These results suggest strongly that some plastid proteins are synthesized upon slug cell ribosomes (26). If this is the case, the genetic information coding for these proteins must somehow be present in the molluscan DNA or must be acquired by all the slugs from the alga in every generation. Our findings presented below suggest a vehicle for this transfer of genetic information from the alga to the slug.

In 1990, as part of an ongoing ultrastructural study of morphological changes in the fine structure of the dying slugs from a population on Martha's Vineyard, Massachusetts, we discovered viruses in digestive cells and hemocytes (29; also Fig. 1). Every year since then, every animal examined near the end of the life cycle has had viruses present, whether it was fixed within hours of collection from the field or maintained for months in our aquarium. No evidence of viruses has been found in any slug earlier in the year, except occasionally within the confines of the plastid (Fig. 1E, see below).

Viruses in the spring slugs are present in the nucleus and cytoplasm of several cell types. They appear to be assembled in the nucleus, then move into the cytoplasm, and finally bud out either into the extracellular space or into a vacuole (Fig. 1A). The diameters of the icosahedral viral capsids in the nuclei average 89 nm (±1.0); but in the cytoplasm, after they have picked up an envelope, they are 109 nm (±1.6) (Fig. 1B). The shapes and sizes of the capsids and envelopes are very similar to those in the Retroviridae, but other viral types have similar dimensions, and known retroviruses are not assembled in the nucleus (30, 31). In addition to these larger viruses, some chloroplasts within the cells of spring slugs contain structures (diameter = 20 nm ± 0.4) that could either be smaller viruses or viral cores. These particles occur loosely collected in areas between the plastid membranes (Fig. 1C). They also occur as particle clumps in the cytoplasm (Fig. 1D), sometimes near material that could be the remnants of chloroplasts. In some instances, in a few fall animals, these particles are present in crystalline arrays (Fig. 1E). Ribulose-1,5-bisphosphate-carboxylase oxygenase (RuBisCO) occurs in crystalline form in some plant plastids fixed under hyperosmotic conditions (32, 33). However, the particles in such RuBisCO crystals are much smaller and usually lack a visual substructure (32, 33), whereas the array particles in the symbiotic plastids in *Elysia* clearly have a substructure (Fig. 1E). The particle arrays in the *E. chlorotica* plastids are more reminiscent of mosaic viruses in plants (34), although almost nothing is known about such crystals in the viruses and plastids of algae. In summary, the morphology suggests that either more than one viral type is present in the slug cell and captured plastids, or we have found several stages of a single viral type.

In addition to the morphology, we have some biochemical information about the identity of the viruses. Using differential and sucrose-gradient centrifugation, we have isolated a fraction from slug homogenates at a density of 1.18 g/ml that has reverse transcriptase (RT) activity. This activity, which is considered diagnostic for retroviruses, is two orders of magnitude higher in spring slugs than in slugs tested in the fall (Fig. 2). In addition, the RT activity of fall animals, but not spring animals, is inhibited by rifampicin (Fig. 3). This inhibition indicates that the activity measured

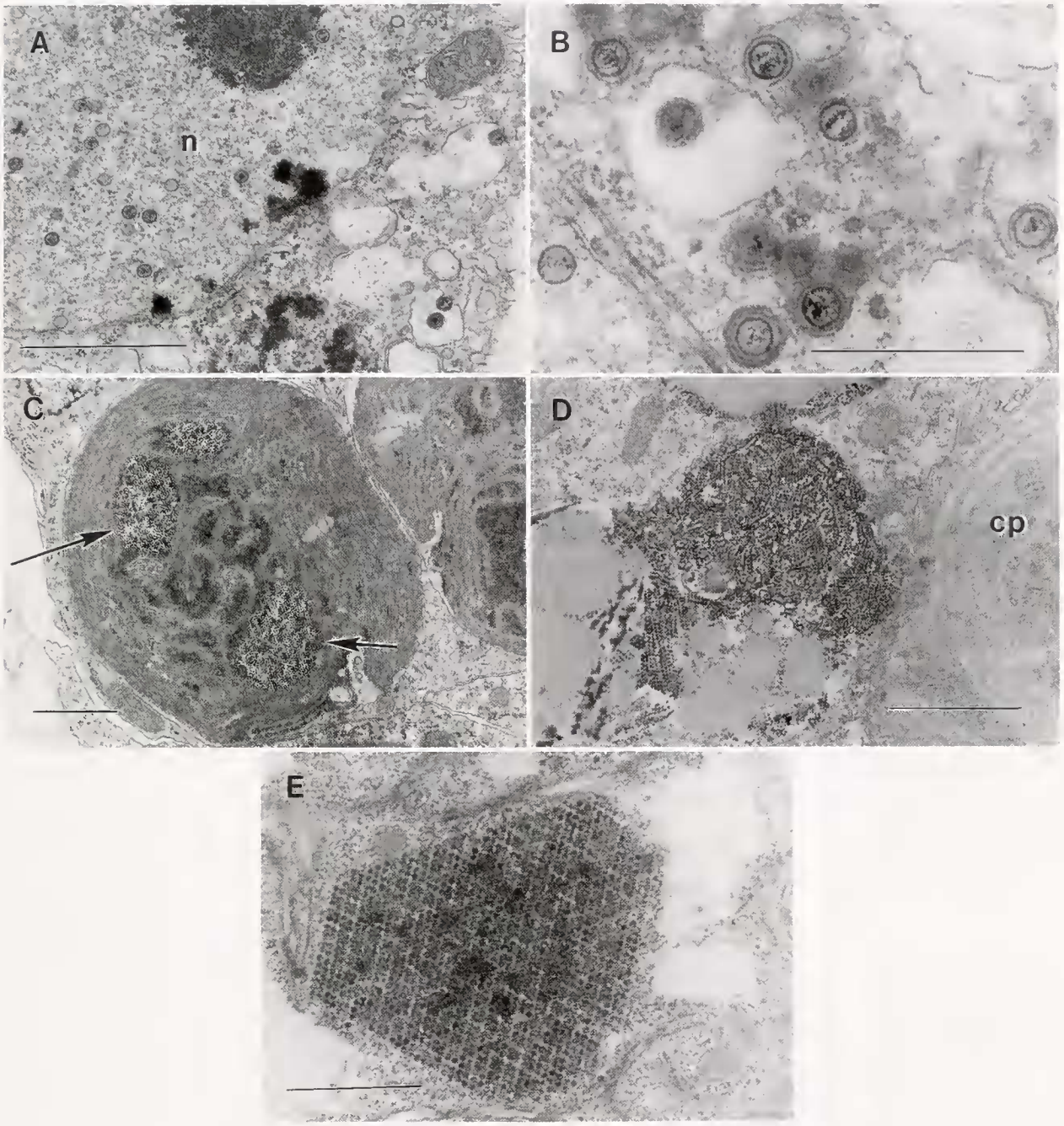


**Figure 1.** Electron micrographs of viruses in *Elysia chlorotica*. (A) Viral particles in various stages of maturation are present in the nucleus (n) and cytoplasm of a hemocyte from a dying slug (magnification = 33,750×, scale bar = 1 μm). (B) Higher magnification (85,000×, scale bar = 0.5 μm) of viruses budding into cytoplasmic vacuoles. Icosahedral shape and double envelopes of the mature virus are apparent. (C) Viral aggregates (arrows) within the symbiotic chloroplast of a spring *Elysia* (magnification = 16,880, scale bar = 1.0 μm). (D) Viral particles very similar in appearance to those in (C) located in the cytoplasm of a chloroplast (cp)-containing digestive cell of a spring slug. The diffuse, gray areas in the cytoplasm are lipid produced by the plastid (magnification = 27,000, scale bar = 1.0 μm). (E) Viral crystal contained in a symbiotic chloroplast from a fall slug (magnification = 54,000, scale bar = 0.5 μm).

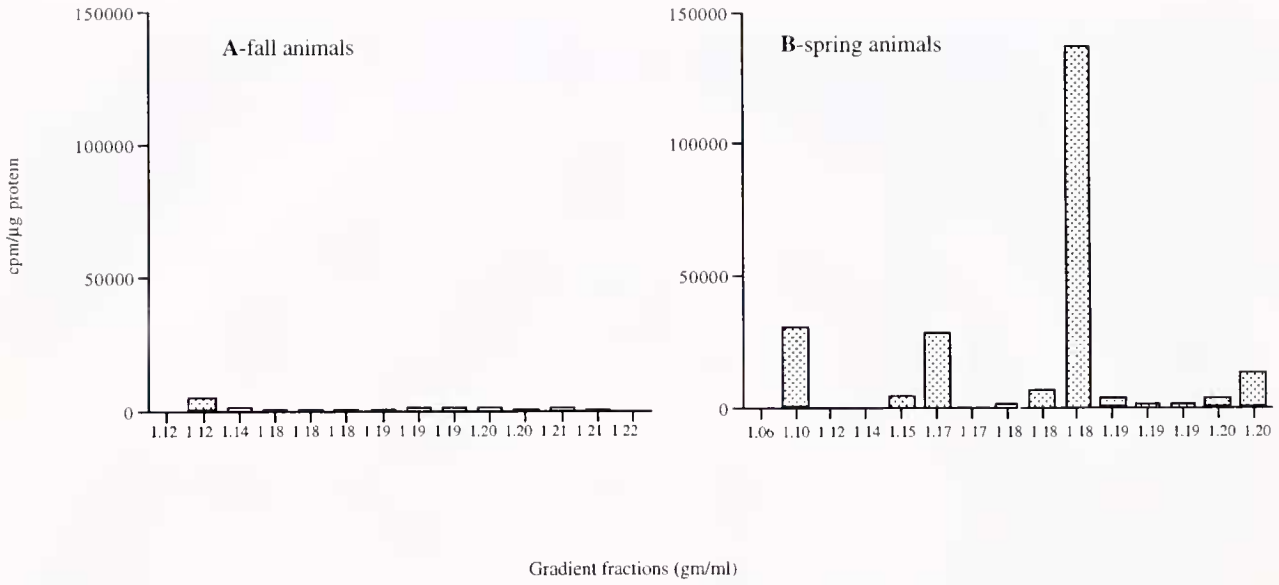


**Figure 2.** Comparison of reverse transcriptase activity in sucrose-gradient fractions from a typical extract of fall slugs (A) and spring slugs (B). The results are essentially the same whether the animals were freshly collected in the spring or collected in the fall and overwintered in aquaria.

in the fall animals is due to DNA dependent-RNA polymerase (which utilizes the same substrates in the RT assay) rather than RT, and confirms the absence of viruses in the fall animals. Taken together, the morphology, the buoyancy, and the presence of RT all suggest that a retro-like virus is present in the cells of the dying slugs.

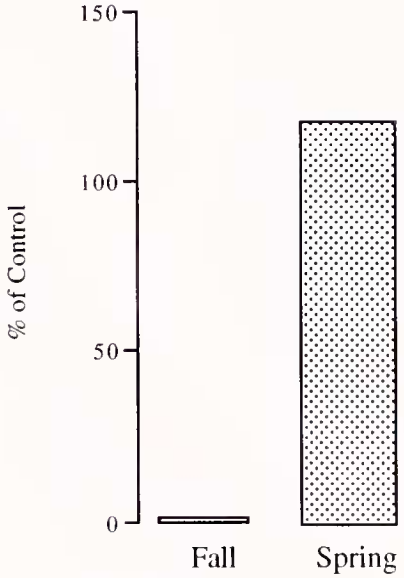


**Figure 3.** Rifampicin inhibits reverse transcriptase activity. Enzyme activity was assayed in pooled gradient fractions with densities from 1.16 to 1.18 g/ml prepared from fall and spring slugs. The effect of the inhibitor is expressed as a percentage of control values.

The relationships between the nuclear, cytoplasmic, and plastid viruses are not known at present. However, for the last 9 years, the viruses have been found in every dying slug examined. Since some of the slugs had been maintained in the laboratory, in aquaria containing artificial seawater and with no access to *Vaucheria* for 8 months before the viruses appeared, the infection is unlikely to have been opportunistic. Instead, the results suggest either that the demise of the entire population is caused by an endogenous virus or that the virus can be expressed only as the defense systems of the aging animals begin to fail. Furthermore, if the effect on the life cycle is due to a retrovirus, as our data suggest, then the viral genome is probably transmitted to the next generation of slugs in the molluscan DNA. Infection of germ cells by retroviruses produces endogenous proviruses that are inherited as Mendelian genes (33). Alternatively, the viruses might enter all of the slugs *via* the sequestered chloroplasts, either as part of the plastid genome or as constituted viral particles. In either case, viral expression is coincident with increases in environmental temperature, at least in the field slugs. Both the onset of egg laying and the death of the population are associated with the rise of water temperatures in the spring. We can delay the demise of the slugs by maintaining them at very cold temperatures (2°–5°C), but eventually—6–8 weeks after the warmer-maintained slugs have died—the cooled slugs also die with viruses in their cells, indicating that temperature is not the only expression stimulus.

It will take some time to sort out both the types of viruses involved here and the molecular relationships between the

slugs, the algae, the plastids, and the viruses. Nevertheless, the nine-year, annual occurrence of the association between population demise and viral expression at the end of the *E. chlorotica* life cycle strongly suggests that the virus has a role in regulating this coincidence. Indeed, we speculate that the viral infection may have caused the transfer, from the alga to the slug, of algal genes that allow the molluscan cells to assist in plastid maintenance. Although the transfer, integration, and expression of such a group of genes between species should succeed only rarely, those successes that did occur should have profound, immediate, heritable effects on the phenotype of the infected species. Such heritable effects must certainly be associated with the mechanism of the widely accepted endosymbiotic origin of intracellular organelles such as mitochondria and chloroplasts; a variety of genes must have been transferred from the symbiont into the host cell nucleus to consummate such a relationship. In addition, a retrovirus as a gene transmission vehicle might have merit as a hypothesis to explain genetic similarities between distantly related or unrelated species (*e.g.*, 35) and is the basis of some genetic therapies (36). If a successful interspecies gene transfer between an alga and a slug mediated by an endogenous virus could be demonstrated in the case of *E. chlorotica*, then an exemplary mechanism for this process would have been provided.

## Methods

### *Viral isolation*

The fractionation procedure was carried out using autoclaved equipment. All reagents were molecular biological grade (DNAase-, RNAase-, and protease-free; from Sigma Chemicals, unless otherwise noted) and filtered (0.2 $\mu$m pore). Approximately 3.0 g of *E. chlorotica* was homogenized in an ice-cold buffer (450 m*M* NaCl, 1.0 m*M* EDTA, 5.0 m*M* 3-[*N*-morpholino]propane-sulfonic acid (MOPS), 2.3 $\mu$*M* leupeptin, 1.0 m*M* dithiothreitol (DTT), 500 $\mu$*M* phenylmethylsulfonyl fluoride (PMSF), pH 7.5) containing the mucolytic agent *n*-acetyl cysteine (500 m*M*), which is necessary to disperse the copious mucus produced by the slug (26). The homogenate was filtered through six layers of cheesecloth, then through one layer of Miracloth (Calbiochem), and finally through two layers of Miracloth. The filtrate was centrifuged for 5 min at 4300 $\times$ *g* (4°C), and the supernatant was centrifuged at 20,000 $\times$ *g* for 30 min (4°C). The supernatant from the second spin was layered over a 20% sucrose cushion and centrifuged at 180,000 $\times$ *g* for 2 h (4°C) in a swinging bucket rotor. The supernatant was discarded, and the pellet was resuspended in ice-cold homogenization buffer (without *n*-acetyl cysteine). This suspension was layered on the top of a 15%–50% continuous sucrose gradient and centrifuged at 180,000 $\times$ *g* for 43 h (4°C).

The gradients were then fractionated by piercing the bottom of the centrifuge tube and raising the gradient out of the tube with 65% sucrose. Twenty 600-$\mu$l fractions were collected, and the density of each was determined by weighing 50 $\mu$l with an analytical balance. The sucrose in each fraction was then diluted with homogenization buffer (without *n*-acetyl cysteine), and each fraction was centrifuged a final time at 180,000 $\times$ *g* for 2 h. The supernatants from this last spin were discarded, and RT assays (see below) and protein assays (37) were run on the pellets.

### *Reverse transcriptase assay*

The final pellets (above) were treated with a detergent buffer (50 m*M* Tris-HCl, 5 m*M* KCl, 0.2 m*M* EDTA, and 0.02% Triton X-100, pH 8.2). Fifteen-microliter aliquots of this digest were added last to 50 $\mu$l of buffer (100 m*M* Tris-HCl, 200 m*M* KCl, 10 m*M* $MgCl_2$, pH 8.2), 6 $\mu$l 100 m*M* DTT, 9.75 $\mu$l 100 m*M* thymidine triphosphate (TTP), 1.25 $\mu$l RNA-guard (Pharmacia), 1.0 $\mu$l poly(rA)-p(dT) (Pharmacia), and 10 $\mu$Ci of $^{32}$P-TTP (ICN, 10 $\mu$Ci/$\mu$l). The final volume was adjusted to 100 $\mu$l with buffer, as necessary, to compensate for $^{32}$P half life. This solution was mixed and incubated at 37°C for 65 min on a shaker table. The reaction was terminated by adding 30 $\mu$l of 10 m*M* EDTA in 5% TCA and placing the reaction mixture on ice for 20 min. DNA was then precipitated by the addition of 9 $\mu$l of 72% TCA, and the precipitate was pelleted by centrifugation at 6610 $\times$ *g* for 10 min. The pellet was washed three times in 5% TCA, the final pellet dissolved in 0.1 *N* NaOH, and the radioactivity determined by scintillation counting. The protein concentration of a separate aliquot was determined, and RT activity was expressed as counts per minute per microgram (cpm/$\mu$g) protein (38).

### *Electron microscopy*

Small pieces of tissue were prepared for microscopy by fixation in 2% glutaraldehyde in 0.15 *M* cacodylate-0.58 *M* sucrose buffer (pH 7.3) at 900 mosm. The tissue pieces were post-fixed in 1.0% $OsO_4$ in the same buffer followed by 2.0% aqueous uranyl acetate. The fixed tissue was dehydrated in an ethanol series, infiltrated with propylene oxide, and embedded in Spurr's medium. Silver sections were cut with an ultramicrotome (Reichert), mounted on 75 $\times$ 300 mesh copper grids, and stained with uranyl acetate and lead citrate. The sections were viewed and photographed with a transmission electron microscope (Zeiss EM 10).

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